



04-21-08

Atty. Dkt. No. 054769-2001

AF

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Chiang, et al.

Title: FLUORESCENCE ENERGY
TRANSFER BY COMPETITIVE
HYBRIDIZATION

Appl. No.: 09/031,087

Filing Date: February 26, 1998

Examiner: Tung, J.

Art Unit: 1637

Conf. No.: 8207

CERTIFICATE OF EXPRESS MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service's "Express Mail Post Office To Addressee" service under 37 C.F.R. § 1.10 on the date indicated below and is addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

EV 955413757 US

April 18, 2008

(Express Mail Label Number)

(Date of Deposit)

Vanessa E. Agha

(Printed Name)

(Signature)

APPEAL BRIEF TRANSMITTAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith are the following:

☒ Appeal Brief (17 pages) with three attachments.☒ Return Postcard.☒ The fee required for additional claims is calculated below:

	Claims As Amended		Previously Paid For		Extra Claims Present		Rate		Additional Claims Fee
Total Claims:	19	-	22	=	0	x	\$50.00	=	\$0.00
Independent Claims:	1	-	3	=	0	x	\$200.00	=	\$0.00
First presentation of any Multiple Dependent Claims: + \$360.00 =									\$0.00
CLAIMS FEE TOTAL =									\$0.00

[X] The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17 or § 41.20, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date 04/18/2008

By Barry Wilson

FOLEY & LARDNER LLP
Customer Number: 30542
Telephone: (858) 847-6722
Facsimile: (858) 792-6773

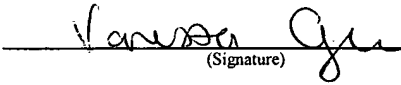
Richard J. Warburg (Reg. no. 32,327)
Attorney for Applicant
By Barry S. Wilson (Reg. No. 39,431)



PATENT
054769-2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Chiang, et al.
Title: FLUORESCENCE ENERGY
TRANSFER BY COMPETITIVE
HYBRIDIZATION
Appl. No.: 09/031,087
Filing Date: February 26, 1998
Examiner: Tung, J.
Art Unit : 1637
Conf. No.: 8207

CERTIFICATE OF EXPRESS MAILING	
I hereby certify that this correspondence is being deposited with the United States Postal Service's "Express Mail Post Office To Addressee" service under 37 C.F.R. § 1.10 on the date indicated below and is addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.	
EV 955413757 US (Express Mail Label Number)	April 18, 2008 (Date of Deposit)
Vanessa E. Agha (Printed Name)	
 (Signature)	

APPEAL BRIEF

Mail Stop Appeal Brief - Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant (hereinafter "Appellant") hereby appeals the Final Rejection of claims 2-11 and 14-22. This Appeal Brief follows a Notice of Appeal filed June 22, 2007. No fee is believed due in conjunction with this Appeal Brief. The required Appeal Brief fee was paid in conjunction with Appellant's Appeal Brief of December 10, 2007. If this fee is incorrect or if any additional fees are due in this regard, please charge or credit our Deposit Account No. 19-0741 for the appropriate amount.

Table of Contents

Table of Authorities	3
Real Party in Interest.....	4
Related Appeals and Interferences.....	4
Status of Claims	4
Status of Amendments	4
Summary of Claimed Subject Matter	4
Grounds for Rejection to be Reviewed on Appeal	5
Argument	5
1. Appellant's claimed invention.	6
2. Rejection of claims 2-11 and 19-22 under 35 U.S.C. § 103(a).....	6
2.1 <i>Tyagi et al.</i>	7
2.2 <i>Diamond et al.</i>	8
2.3 <i>Tyagi et al. teaches away from the use of bimolecular probes in amplification reactions.</i>	8
2.4 <i>The Examiner has failed to make a prima facie case of obviousness.</i>	9
3. Rejection of claims 14-18 under 35 U.S.C. § 103(a).....	12
Conclusion	12
Appendix A: Claims Appendix.....	14
Appendix B: Evidence Appendix	17
Appendix C: Related Proceedings Appendix	18

Table of Authorities

CASES

<u>Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc.,</u> 796 F.2d 443, 448, 230 USPQ 416 (Fed. Cir. 1986)	11
<u>Graham v. John Deere Co. of Kansas City,</u> 383 U.S. 1, 86 S.Ct. 684 (1966).....	10
<u>In re Fritch,</u> 972 F.2d 1260, 23 USPQ2d 1780 (Fed. Cir. 1992)	11
<u>In re Geisler,</u> 116 F.3d 1465, 43 USPQ2d 1362, (Fed. Cir. 1997)	11
<u>In re Haruna,</u> 249 F.3d 1327, 58 USPQ2d 1517 (Fed. Cir. 2001)	11
<u>In re Kahn,</u> 441 F.3d 977, 988, 78 U.S.P.Q.2d 1329, 1336 (Fed. Cir. 2006)	9
<u>In re Malagari,</u> 499 F.2d 1297, 182 USPQ 549 (CCPA 1974)	11
<u>In re Wesslau,</u> 353 F.2d 238, 241, 147 USPQ 391, 393 (CCPA 1965)	11
<u>KSR Int’l Co. v. Teleflex Inc.,</u> 127 S.Ct. 1727, 1741 (2007)	9, 10

Real Party in Interest

The real party in interest in this appeal is Quest Diagnostics Investments Incorporated, which is the assignee of the present application.

Related Appeals and Interferences

None.

Status of Claims

Claims 1 and 12-13 have been canceled.

Claims 2-11 and 14-22 are pending and under examination in the application.

Claims 2-11 and 14-22 are the subject of this appeal.

Status of Amendments

The last claim amendments were presented in Appellant's Amendment and Reply Under 37 C.F.R. 1.111 of January 18, 2007. The claim amendments contained therein have been entered, examined, and are appealed herein. No other amendments or submissions are pending in the application.

Summary of Claimed Subject Matter

The present invention provides methods for monitoring oligonucleotide production during an amplification reaction. Specifically, the claimed method uses a two probe system in which a first probe contains a fluorophore, a second probe contains a quencher molecule and the two probes are capable of hybridizing, causing quenching of the fluorescent signal. A target oligonucleotide produced by the amplification reaction, having a sequence complementary to one of the probes, competitively hybridizes to that probe causing separation of fluorophore-containing probe and the quencher-containing probe. This competitive hybridization results in increased fluorescence as the fluorescent signal is no longer quenched. Thus, the change in fluorescence over the course of an amplification reaction may be used to monitor the production of the target oligonucleotide.

Claim 20, the sole pending independent claim, encompasses a method for monitoring nucleic acid amplification by amplifying a target nucleic acid in the presence of a first oligonucleotide probe comprising a fluorophore and a second oligonucleotide probe comprising a quencher molecule, wherein the first and second probes are not equal in length and are cable of hybridizing to each other. Amplification of the target nucleic acid is monitored by detecting fluorescence during the amplification reaction, wherein an increase in fluorescence correlates with target nucleic acid amplification. Support for claim 20 is found in the Specification at page 2, lines 1-19, page 4, line 9 through page 5, line 25, page 6, line 10 through page 7, line 17, page 7, line 23 through page 8, line 14, and page 9 line 11 through page 10, line 9.

Claims 14-18 each depend from claim 20 and further require the use of probes and primers having the particular nucleotide sequences of SEQ ID NOs: 3, 4, 1, and 2, respectively, relating to the hepatitis C virus (HCV). Support for claims 14-18 is found in the Specification at page 2, lines 1-19 and page 8, Tables I and II.

Grounds for Rejection to be Reviewed on Appeal

1. Claims 2-11 and 19-22 stand finally rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Tyagi et al. (U.S. Patent 6,103,476) in view of Diamond et al. (U.S. Patent 4,766,062).

2. Claims 14-18 stand finally rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Tyagi et al. (U.S. Patent 6,103,476) in view of Diamond et al. (U.S. Patent 4,766,062 and Hiroaki et al. (EP 0461 863).

Argument

All pending claims stand rejected for obviousness over the basic combination of Tyagi et al. in view of Diamond et al. As discussed in more detail below, the Examiner ignores a clear and unequivocal teaching away by Tyagi et al. against the use of bimolecular probes (i.e., two individual but complementary probes) for detecting target nucleic acids in amplification reactions such as PCR. It is exactly the use of bimolecular probes that makes Applicant's claimed invention unobvious. For this and other reasons, the Examiner has engaged in an impermissible hindsight reconstruction of Appellant's invention. The Examiner has merely

pointed to the claim elements in prior art as evidence of obviousness but has failed to demonstrate that the prior art provides a motivation to combine those elements or provides a reasonable expectation of success in their combination. Thus, the Examiner has failed to make a *prima facie* case of obviousness.

1. Appellant's claimed invention.

The claimed invention is directed to a method for monitoring nucleic acid amplification, for example, during PCR. The method requires the use of two probes (i.e., a first probe and a second probe) which contain a fluorophore and a quencher molecule, respectively. The "bimolecular" probes are capable of hybridizing in a manner that causes fluorescence quenching. At least one of the probes is also capable of hybridizing to the target nucleic acid. When this latter hybridization occurs, the fluorophore and quencher molecule are spatially separated because the two probes are no longer hybridized to each other. This results in an increased amount of detectable fluorescence that is related to the amount of target nucleic acid present.

As discussed in more detail below, a key feature of the claimed invention is the use of two probe molecules (i.e., a bimolecular probe), each containing either the fluorophore or the quencher molecule, as opposed to a single probe molecule (i.e., a unimolecular probe) containing both the fluorophore and quencher.

2. Rejection of claims 2-11 and 19-22 under 35 U.S.C. § 103(a).

Appellant respectfully traverses the final rejection of claims 2-11 and 19-22 under 35 U.S.C. § 103(a) as allegedly being obvious over Tyagi et al. (U.S. Patent 6,103,476) in view of Diamond et al. (U.S. Patent 4,766,062).

The Examiner alleges that Tyagi et al. provide methods for detecting target nucleic acids in a variety of assays, including monitoring the progress of an amplification and other reactions. Final Office Action mailed March 22, 2007, at p. 2, ¶ 3. The Examiner acknowledges that the Tyagi probes are different from Appellant's probes. Final Office Action at p. 2, ¶ 4 ("Tyagi et al. do not disclose the probe, which has the features recited in claims 20, 22, and 3-10."). However, the Examiner attempts to remedy this deficiency by relying on teachings of Diamond et al.,

which the Examiner alleges discloses probes having the same features as recited in claims 20, 22, and 3-10. Final Office Action at p. 2, ¶ 5.

In asserting this rejection, the Examiner misinterprets the teachings of Tyagi et al., as they relate to Appellant's claimed invention. Furthermore, by alleging that there is a motivation to combine the teachings of Tyagi et al. and Diamond et al., the Examiner ignores Tyagi's clear teaching away from the use of bimolecular probes for monitoring a nucleic acid amplification reaction, as presently claimed.

2.1 Tyagi et al.

Tyagi et al. provide methods for detecting target nucleic acids in a variety of assays, including monitoring the progress of an amplification and other reactions. Tyagi et al. at col. 3, l. 51 through col. 4, l. 21. The methods are based on providing a "unitary probe" which contains (i) a target complementary region, (ii) an affinity pair flanking the target complementary region, and (iii) interactive label moieties. Tyagi et al. at col. 4, l. 59 through col. 5, l. 2. The target complementary region encodes a nucleotide sequence complementary to the target nucleic acid which serves as the basis for target-specific detection. The interactive label moieties (e.g., fluorophore and quencher) are pairs of molecules in which one member can alter a physically-detectable property of the other member. Tyagi et al. at col. 5, ll. 13-26. The affinity pair (e.g., complementary nucleic acid sequences) holds the unitary probe in a "closed conformation" in the absence of target nucleic acid such that the interactive label moieties are held in close physical proximity. The "open conformation" of the unitary probe occurs in the presence of target nucleic acid which disrupts the affinity pair binding, causing physical separation of the interactive label moieties. Tyagi et al. at col. 5, ll. 13-63.

Despite the potentially confusing terminology, Tyagi's "unitary probes" are designed to act as a functional unit, and may be unimolecular or bimolecular. Tyagi et al. at col. 4, ll. 59-62. In a bimolecular construction, the Tyagi probes consist of a pair of oligonucleotides, each containing a different target complementary sequence and an "arm" portion complementary to the arm portion of the other oligonucleotide. Tyagi et al. at col. 5, ll. 2-4. Together, hybridization of the arm portions form a stem which holds the fluorophore and quencher in close proximity, sufficient for quenching. Tyagi et al. at col. 6, ll. 20-26, col. 9, ll. 59-65, and Figure

1. The interactive label moieties are separated when one or both of the target complementary regions (i.e., probes) is bound to the target nucleic acid.

2.2 Diamond et al.

The Examiner acknowledges that the biomolecular probes of Tyagi et al. are not identical to those required in Appellant's claimed method. Specifically, the Examiner acknowledges that the Tyagi probes are the same as Appellant's probes except that Tyagi et al. do not disclose probes of unequal length. For this, the Examiner turns to Diamond et al. which disclose bimolecular probes of various lengths. Appellant does not disagree with this characterization of Diamond et al.

2.3 Tyagi et al. teaches away from the use of bimolecular probes in amplification reactions.

According to Tyagi et al., the choice of whether to use unimolecular or bimolecular probe depends upon the particular conditions under which the target nucleic acid is to be detected. Here, Tyagi et al. are very clear.

[F]or assays that include a step or steps that may separate the affinity pair in a target-independent manner, only unimolecular probes are suitable.

Tyagi et al. at col. 6, ll. 32-34 (emphasis added).

Tyagi et al. elaborates on the prohibition against the use of bimolecular probes:

Bimolecular probes, as stated above, are not suitable for use in any reaction, e.g., PCR, in which the affinity pair would be separated in a target-independent manner.

Tyagi et al. at col. 6, l. 67 through col. 5, l. 3 (emphasis added). .

On the desirability of using unimolecular probes for amplification reactions including PCR, Tyagi et al. teach:

Unimolecular probes with interactive labels according to the invention are particularly useful in assays for tracking polymerase chain reactions, since the probes according to this invention can open and close with a speed that is faster than the speed of thermal cycling.

Tyagi et al. at col. 22, l. 66 through col. 23, l. 3 (emphasis added).
See, also, Tyagi et al. at col. 20, ll. 47-49.

Tyagi et al. provide a sound scientific rationale supporting their admonition against the use of bimolecular probes in amplification reactions. Tyagi et al. teach:

These strand displacement probe complexes have drawbacks. The mechanisms is two-step, in that the probe complex must first bind to the target and then strand-displacement, via branch migration, must occur before a target is recognized and a signal is generated. Bimolecular probe complexes are not reported to form with high efficiency, resulting in probe preparations wherein the majority of the target binding regions may not be annealed to a labeled strand. This may lead to competition between label-bearing and label-free target binding regions for the same target sequence... Moreover, the displaced labeled strand may need to be separated from the unhybridized probe complexes before a signal may be detected.

Tyagi et al. at col. 3, ll. 11-24 (emphasis added).

Thus, the teachings of Tyagi et al. are clear: bimolecular probes should not be used in assays, such as PCR, in which the probes may be separated in the absence of target nucleic acid. This is a clear teaching away from Appellant's claimed method because, as discussed above, the claimed method requires the use of bimolecular probes for monitoring an amplification reaction.

2.4 The Examiner has failed to make a *prima facie* case of obviousness.

An invention is unpatentable as obvious if the differences between the patented subject matter and the prior art would have been obvious at the time of invention to a person of ordinary skill in the art. Rejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness. KSR Int'l Co. v. Teleflex Inc., 127 S.Ct. 1727, 1741 (2007) (quoting In re Kahn, 441 F.3d 977, 988, 78 U.S.P.Q.2d 1329, 1336 (Fed. Cir. 2006)). Thus, in order to establish a *prima facie* case of obviousness, it is necessary for the Examiner to identify the reasons why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed. The proper analysis when determining obviousness includes consideration of the scope and content of the prior art; the level of ordinary skill in the prior art; the differences between the claimed invention and the prior art; and objective evidence

of nonobviousness. KSR at 1734, citing Graham v. John Deere Co. of Kansas City, 383 U.S. 1, 86 S.Ct. 684 (1966).

In asserting this rejection, the Examiner concludes that it is *prima facie* obvious to use Diamond's bimolecular probes of unequal length in the method of Tyagi et al. to monitor amplification reactions. Final Office Action at p. 3, ¶ 1. However, the Examiner has failed to substantively address the teaching away by Tyagi et al. The Examiner's sole rationale for ignoring Tyagi's clear admonition against the use of bimolecular probes for monitoring amplification reactions is merely that the probes of Tyagi et al. and Diamond et al. are different. Final Office Action at paragraph bridging pp. 3-4. The Examiner fails to identify the specific difference or how it serves to refute Tyagi's clear teachings. The Examiner states:

[T]he physiochemical rational discussed by Tyagi et al. (See column 3, lines 11-18) is not applicable to the bimolecular probe disclosed by Tyagi et al. because the bimolecular probe as discussed in column 3, lines 11-18 is different from the probes disclosed by Tyagi et al.

Final Office Action at paragraph bridging pp. 3-4 (emphasis added).

Appellant assumes that the Examiner meant to state that the bimolecular probe as disclosed by Tyagi et al. at col. 3, ll. 11-18 is different from the probes disclosed by Diamond et al. This argument is clearly specious. As noted above, the Examiner's sole alleged distinction between the probes of Tyagi et al. and those of Diamond et al. and Appellant is that the bimolecular probes of Diamond et al. and Appellant have unequal length, whereas Tyagi et al. provides bimolecular probes having the same length. The Examiner has failed to explain why a mere difference in probe length renders Tyagi's prohibition against the use of bimolecular probes invalid.

A *prima facie* case of obviousness has not been made. Although the Examiner has managed to point to each of the elements of Appellant's claimed invention, the Examiner has failed to demonstrate that the cited prior art provides a motivation to combine those elements in a manner that results in the claimed invention, and the Examiner has failed to demonstrate that the

prior art provides a reasonable expectation of success. This rejection is founded solely on conclusory statements, devoid of sound reasoning or a rational underpinning.

The primary flaw in this rejection is the Examiner's refusal to acknowledge and substantively address Tyagi's teaching away from the use of bimolecular probes to detect target nucleic acids in amplification reactions. Such a teaching away cannot be ignored. The Federal Circuit has mandated that, when considering the scope and content of the prior art, it is necessary to consider the entire teachings of a prior art reference, not just selected pieces to support an obviousness rejection. In re Fritch, 972 F.2d 1260, 1264, 23 USPQ2d 1780 (Fed. Cir. 1992)("[A] prior art reference is relevant for all that it teaches to those of ordinary skill in the art."). "It is impermissible within the framework of section 103 to pick and choose from any one reference only so much of it as will support a given position to the exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one skilled in the art." Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc., 796 F.2d 443, 448, 230 USPQ 416 (Fed. Cir. 1986), quoting In re Wesslau, 353 F.2d 238, 241, 147 USPQ 391, 393 (CCPA 1965). Furthermore, "[a] *prima facie* case of obviousness can be rebutted if the applicant... can show 'that the art in any material respect taught away' from the claimed invention." In re Haruna, 249 F.3d 1327, 1335, 58 USPQ2d 1517 (Fed. Cir. 2001), citing In re Geisler, 116 F.3d 1465, 1469, 43 USPQ2d 1362, 1365 (Fed. Cir. 1997), In re Malagari, 499 F.2d 1297, 1303, 182 USPQ 549, 553 (CCPA 1974).

In sum, Appellant has surprisingly discovered that bimolecular probes are effective for monitoring amplification reactions. This discovery is in sharp contrast to the expectation of Tyagi et al. who specifically and repeatedly teach away from such a method. The Examiner has failed to make a *prima facie* case of obviousness because the cited prior art provides no motivation to make Appellant's claimed method and provides no reasonable expectation that such a method would be successful. The Examiner has presented no evidence or convincing rationale to refute Tyagi's teachings sufficient to render Appellant's claimed invention obvious. The rejection of claims 2-11 and 19-22 is traverse and should be withdrawn.

3. Rejection of claims 14-18 under 35 U.S.C. § 103(a).

Claims 14-18 stand finally rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Tyagi et al. (U.S. Patent 6,103,476) in view of Diamond et al. (U.S. Patent 4,766,062) and Hiroaki et al. (EP 0461863). The Examiner applies Tyagi et al. and Diamond et al. as discussed above and acknowledged that these references do not teach hepatitis C virus (HCV) polynucleotide probes having the sequence of SEQ ID NOs: 3 and 4, or HCV polynucleotide primers having the sequence of SEQ ID NOs: 1 and 2. For these polynucleotides, the Examiner turns to Hiroaki et al. which discloses a portion of the 5'- noncoding region of HCV. The Examiner concludes that it would have been *prima facie* obvious to use probes of SEQ ID NOs: 3 and 4 and primers of SEQ ID NOs: 1 and 2, as found contained within the sequences of Hiroaki et al., in combination with the combined method of Tyagi et al. and Diamond et al., as discussed above. Appellant respectfully traverses this rejection.

Claims 14-18 depend from claim 20. As discussed above, claim 20 is unobvious over the combination of Tyagi et al. and Diamond et al. The claimed method requires the use of two separate probe molecules (i.e., a bimolecular probe) for monitoring amplification reactions. Tyagi et al. provides repeated and strong teachings away from the use of bimolecular probes for this purpose. As discussed above, Diamond et al. is applied by the Examiner to teach bimolecular probes having unequal lengths, but does not refute the teaching away provided by Tyagi et al.. Hiroaki et al. is applied only for the purpose of providing HCV-specific polynucleotides. Thus, Hiroaki does nothing to remedy this deficiency in the Examiner's *prima facie* case. For this reason alone, the rejection of claims 14-18 is traversed and should be withdrawn.

Conclusion

For the reasons discussed above, Appellant respectfully submits that claims 2-11 and 14-22 are in condition for allowance, and respectfully request that the rejections be withdrawn or reversed, and that the claims be allowed to issue.

Respectfully submitted,

Date: 04/18/2008

FOLEY & LARDNER
P.O. Box 80278
San Diego, CA 92138-0278
(858) 847-6700 (Voice)
(858) 792-6773 (Fax)

By: Barry Wilson

Richard J. Warburg, Reg. No. 32,327
By Barry Wilson, Reg. No. 39,431
Attorneys for Applicant

Appendix A: Claims Appendix

1. (Cancelled).
2. (Previously presented) The method of claim 20 wherein the amplification is carried out using a thermostable nucleic acid polymerase.
3. (Previously presented) The method of claim 20 wherein the fluorophore on the first probe and the quencher molecule on the second probe are on complementary base pairs.
4. (Previously presented) The method of claim 20 wherein the fluorophore and quencher molecules are within about 1 to 3 hybridized base pairs of each other.
5. (Previously presented) The method of claim 20 wherein the fluorophore and quencher molecules are within 3 or more hybridized base pairs of each other.
6. (Previously presented) The method of claim 20 wherein the fluorophore is on the 5' terminal nucleotide of the first probe and the quencher is on the 3' terminal nucleotide of the second probe.
7. (Previously presented) The method of claim 20 wherein the fluorophore is on the 3' terminal nucleotide of the first probe and the quencher is on the 5' terminal nucleotide of the second probe.
8. (Previously presented) The method of claim 20 wherein the second probe is shorter than the first probe by deletion of 3 or 3' terminal nucleotides from the nucleotide sequence of the first probe.
9. (Previously presented) The method of claim 20 wherein the second probe is shorter than the first probe by deletion of 3 or more 3' terminal nucleotides from the nucleotide sequence of the first probe.
10. (Previously presented) The method of claim 20 wherein the second probe is shorter than the first probe by deletion of 3 or more 5' terminal nucleotides, and deletion of 1 or more 3' terminal nucleotides of the first probe.

11. (Previously presented) The method of claim 20 wherein the first and second probes have a dissociation temperature difference of 2 degrees or more.

12-13. (Cancelled).

14. (Previously presented) The method of claim 20 wherein the first probe has the sequence of SEQ ID NO. 3.

15. (Previously presented) The method of claim 20 wherein the first probe has the sequence of SEQ ID NO. 4.

16. (Previously presented) The method of claim 20 wherein the amplification method is the polymerase chain reaction and wherein a primer for use in the polymerase chain reaction has the sequence of SEQ ID NO. 1.

17. (Previously presented) The method of claim 20 wherein the amplification method is the polymerase chain reaction and wherein a primer for use in the polymerase chain reaction has the sequence of SEQ ID NO. 2.

18. (Previously presented) The method of claim 20 wherein the target polynucleotide is a polynucleotide comprising the hepatitis C virus genome or segment thereof.

19. (Previously presented) The method of claim 20 wherein the method of amplification is selected from the group consisting of ligase chain reaction, gap ligase chain reaction, transcription mediated amplification, nucleic acid sequence based amplification and strand displacement amplification.

20. (Previously presented) A method for monitoring nucleic acid amplification comprising:

amplifying a target nucleic acid and monitoring said target nucleic acid during said amplification using a first oligonucleotide probe and a second oligonucleotide probe, said first probe;

i) hybridizes to said target nucleic acid;

ii) comprises a fluorophore; and

iii) is not equal in length to said second probe;
said second probe;
i) hybridizes to said first probe; and
ii) has a quencher molecule which quenches said first probe fluorophore when said
first and second probes are hybridized to each other; and
detecting fluorescence of said first probe fluorophore to monitor amplification, wherein an
increase in fluorescence correlates with amplification.

21. (Previously presented) The method of claim 20 wherein the amplification method
includes the use of a primer pair that flanks the first and second probe.

22. (Previously presented) The method of claim 20 wherein the longer probe binds
preferentially to the target polynucleotide and when preferentially bound to the target
polynucleotide the fluorescence intensity of the fluorophore is greater than the fluorescence
intensity of the fluorophore when hybridized to the second probe.

Appendix B: Evidence Appendix

1. Diamond et al, U.S. Patent 4,766,062.
2. Hiroaki et al., European Patent Publication EP 0461863.
3. Tyagi et al., U.S. Patent 6,103,476.

Appendix C: Related Proceedings Appendix

None.



Europäisches Patentamt
European Patent Office
Office européen des brevets



⑪ Publication number : **0 461 863 A1**

⑫

EUROPEAN PATENT APPLICATION

⑫ Application number : **91305270.0**

⑮ Int. Cl.⁵ : **C07H 21/04, C12Q 1/70,
C12Q 1/68, C12N 15/51,
C12P 19/34**

⑭ Date of filing : **11.06.91**

⑰ Priority : **12.06.90 JP 153402/90**

⑱ Date of publication of application :
18.12.91 Bulletin 91/51

⑲ Designated Contracting States :
BE CH DE DK FR GB IT LI NL

⑳ Applicant : **IMMUNO JAPAN INC.
4-28-14-701, Ogikubo Suginami-Ku
Tokyo 167 (JP)**

㉑ Inventor : **Okamoto, Hiroaki
3132-24, Yakushi-ji, Minami Kawachi Machi
Kawachi-Gun, Tochigi-ken (JP)
Inventor : Nakamura, Yetsuo
4-28-14-701, Ogikubo, Suginami-ku
Tokyo 167 (JP)**

㉒ Representative : **Arthur, Bryan Edward et al
Withers & Rogers 4 Dyer's Buildings Holborn
London EC1N 2JT (GB)**

㉓ **Oligonucleotide primers, and their application for high-fidelity detection of non-A, non-B hepatitis virus.**

㉔ **The noncoding region, containing 324 nucleotides, of the 5' terminus of non-A, non-B hepatitis virus is disclosed. Also disclosed is the nucleotide sequence of the structural gene which is upstream of the noncoding region. Oligonucleotide primers derived from these regions can be used to detect non-A, non-B hepatitis virus.**

EP 0 461 863 A1

Introduction to the Invention

The present invention concerns high-fidelity detection of non-A, non-B hepatitis virus (hereinafter called NANB hepatitis virus) and oligonucleotide primers used in a detection system for detecting NANB hepatitis virus.

Viral hepatitis of which DNA and RNA have been elucidated include hepatitis A, hepatitis B, hepatitis D and hepatitis E. However, in spite of great efforts by scientists the world over, the causative virus of NANB hepatitis (which is mainly caused by blood borne infection) falls in none of the above groups and has not been isolated.

In 1988, Chiron Corp. reported that it had succeeded in cloning the RNA virus genome of the causative agent of NANB hepatitis (which it termed hepatitis C virus (hereinafter called HCV)) and disclosed part of the nucleotide sequence of HCV. HCV antibody detection systems based on that sequence are now being introduced for screening of blood for transfusion and for diagnosis of patients.

However, the nucleotide sequence disclosed by Chiron Corp. was only part of the NANB hepatitis viral genome. Moreover, it was part of a sequence of relatively little importance. HCV antibody detection systems developed on the basis of that sequence, therefore, fail to provide both sufficient sensitivity and specificity for NANB hepatitis virus and for therapy and prognosis of acute and chronic NANB hepatitis, although such systems have proven their partial association with NANB hepatitis.

More than 95% of posttransfusion hepatitis cases in Japan are NANB hepatitis. There are 280,000 annual estimated cases of this disease. The course of NANB hepatitis is troublesome, with most patients becoming carriers who develop chronic hepatitis. In addition, those patients with chronic hepatitis develop liver cirrhosis and then hepatocellular carcinoma at a fairly high rate over 10 to 20 years. Therefore it is imperative to isolate the virus itself and to develop effective diagnostic reagents enabling earlier diagnosis.

As described earlier, there are significant numbers of patients with acute or chronic NANB hepatitis which can not be diagnosed by the detection systems using Chiron's HCV antibody. For accurate diagnosis of these cases of hepatitis, detection systems for the virus based on elucidation of the viral agent at its gene level is required.

Summary of the Invention

An object of the present invention is to provide a highly sensitive detection system for NANB hepatitis virus at its gene level and oligonucleotide primers used for such system.

Brief Description of the Drawing

Figure 1 shows determination method of nucleotide sequences of NANB hepatitis viral RNA.

Detailed Description of the Invention

For the purpose of elucidation of the NANB hepatitis viral gene, the inventors isolated NANB hepatitis viral RNA from human and chimpanzee carrier sera and determined the nucleotide sequence of the 5' terminus by cloning its cDNA. As a result, the inventors identified that for two different strains the RNA genome had a 5' noncoding region having a sequence of at least 324 nucleotides. This sequence had not been disclosed by Chiron and is totally novel. It was also determined that the nucleotide sequence in this region was highly conserved among different strains. For example, the RNA of strains HC-J1 and HC-J4 (used for the determination of the nucleotide sequence) differed from each other by only three nucleotides.

On the other hand, large differences (or mutations) in nucleotides were identified in other regions of NANB hepatitis viral RNA. When this fact is taken into consideration, it is amazing that the nucleotide sequence is conserved so well in the 5' noncoding region. It was further determined that there were few differences in the nucleotide sequence in the upstream part of the structural gene following the noncoding region. Based on these findings, the inventors discovered that use of oligonucleotide primers derived from these regions would detect, with high sensitivity, NANB hepatitis RNA irrespective of the strain.

The present invention, therefore, concerns a NANB hepatitis virus detection system using oligonucleotide primers having nucleotide sequences corresponding to part of the 5' noncoding region of the viral RNA and/or part of the 5' side of the region coding for the structural protein of the virus.

The primers may contain from about 15 to about 25 nucleotides, preferably 20.

Abbreviations used in this invention are as follows: for RNA, A, G, C and U stand for adenine, guanine, cytosine and uracil respectively; for DNA, A, G, and C indicate the same bases as in RNA and T stands for

thymine; for polypeptides A, R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y and V are respectively the amino acids of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

The inventors have identified that the 5' noncoding region has the following nucleotide sequence (HC-J1 strain):

```

      G G C C A C A C T C   C A C C A T A G A T
      C A C T C C C C T G   T G A G G A A C T A
10    C T G T C T T C A C   G C A G A A A G C C
      T C T A G C C A T G   G C G T T A G T A T
      G A G T G T C G T G   C A G C C T C C A G 100
15    G A C C C C C C C T   C C C G G G A G A G
      C C A T A G T G G T   C T G C G G A A C C
      G G T C A G T A C A   C C G G A A T T G C
20    C A G G A C G A C C   G G G T C C T T T C
      T T G C A T A A A C   C C G C T C A A T G 200
      C C T G C A G A T T   T G G G C C C G C C
25    C C C G C A A G A C   T G C T A G C C G A
      G T A G T G T T G C   G T C G C G A A A G
      G C C T T G T G G T   A C T G C C T G A T
30    A G G C T G C T T C   C G A G T G C C C C 300
      G G G A C G T C T C   G T A C A C C G T G
      C A C C

```

35 The inventors have further determined that the remaining sequence of the 5' noncoding region of the HC-J4 strain is identical to that of HC-J1 (except for the 187th nucleotide A which is replaced by C, the 217th nucleotide C which is replaced by T, and the 226th nucleotide A which is replaced by G). The inventors subsequently developed the highly sensitive detection system for NANB hepatitis virus using oligonucleotide primers corresponding to part of the 5' noncoding region of NANB hepatitis virus. Therefore, any oligonucleotide primer belonging to this defined region of HCV genome may be included in this invention, though replacement of one or more nucleotides is also included.

40 The inventors have also identified that the upstream region coding for the structural protein of the virus following the aforementioned 5' noncoding region was well conserved among the strains. There were only a few differences between the strains. Nucleotide sequences corresponding to part of that region, used as primers, can detect NANB hepatitis virus with high sensitivity.

The upstream region of the structural gene of the NANB hepatitis virus has the following nucleotide sequence (for the HC-J1 strain):

50

55

	ATGAGC	ACGATTCCCA
5	AACCTCAAAG	AAAAAACCAAA
	CGTAACACCA	ACCGTCGCCC
	ACAGGACGTC	AAGTTCCCCGG400
	GTGGCGGTCA	GATCGTTGGT
10	GGAGTTTACT	TGTTGCCCGCG
	CAGGGGCCCT	AGATTGGGTG
	TGGCGCGCAC	GAGGAAGACT
15	TCCGAGCGGT	CGCAACCTCG500
	AGGTAGACGT	CAGCCTATCC
	CCAAGGTGCG	TGGCGCCCGAG
20	GGCAGGACCT	GGCCTCAGCC
	CGGGTACCCT	TGGCCCCCTCT
	ATGGCAATGA	GGCCTGCCGG600
25	TGGCGGGGAT	GGCTCCTGTC
	TCCCCCGTGGC	TCTCGGCCTA
30		
	GTTGGGGCCC	CACGGACCCC
	CGGCGTAGGT	CGCGCAATTT
35	GGGTAAGGTC	ATCGATACCC700
	TCACGTGCGG	CTTCGCCGAC
	CTCATGGGGT	ACATACCGCT
40	CGTCGGCGCC	CCTCTTGCAG
	GCGCTGCCAG	GGCCCTGCCG
	CATGCCGTCC	GGGTTCTCGA800
45	AGACGGCGTG	AACTATGCAA
	CAGGGAACCT	TCCTGGTTGC
	TCTTTCTCTA	TCTTCCTTCT

50 Except for the following differences, the upstream region of strain HC-J4 has the same nucleotide sequence as HC-J1 (numbers in parenthesis show respective differences in the sequence from the 5' noncoding region):

55

5
 10
 A (335), T (339), C (375), C (402),
 T (405), C (430), C (450), G (453),
 T (471), T (501), A (504), T (505),
 G (507), A (510), A (513), C (527),
 T (528), C (531), A (534), G (547),
 T (596), G (597), A (606), A (621),
 C (627), C (633), T (675), C (678),
 T (701), A (705), T (720), T (732),
 T (734), C (753), A (756), G (759),
 T (775), A (780), C (783), T (786),
 G (801), T (829), G (831), C (834),
 C (858), T (859).

15 As far as oligonucleotide corresponding to part of the upstream region of the structural gene of the virus is concerned, also included are nucleotide sequences with a small number of nucleotides different from strains HC-J1 and HC-J4.

This invention includes detection of NANB hepatitis virus by amplification of cDNA of the viral RNA by Polymerase Chain Reaction (hereinafter called PCR) using oligonucleotide primers disclosed herein. PCR is a method well known in this art.

20 Under optimum conditions, using the product of the first amplification of cDNA by PCR as a template, a second amplification by PCR is carried out. In the second PCR amplification, a pair of primers that can be annealed inside the first pair of primers is used.

25 The present invention includes oligonucleotide primers used in the above described detection system. This invention also includes creation of oligonucleotide primers having nucleotide sequences corresponding to part of the 5' noncoding region of NANB hepatitis viral RNA genome (having at least 324 nucleotides), and creation of oligonucleotide primers having nucleotide sequences specific to part of the 5' region coding for the structural protein of the virus.

The following primers are particularly preferable (numbers in parenthesis show positions in sequence from the 5' noncoding region):

30
 #23: T A G A T T G G G T G T G C G C G C G A (450-469 of strain HC-J1),
 #25: T C C C T G T T G C A T A G T T C A C G (807-826),
 35 #32: A C T C C A C C A T A G A T C A C T C C (7- 26),
 #33: T T C A C G C A G A A A G C G T C T A G (46- 65),
 #36: A A C A C T A C T C G G C T A G C A G T (229-248), and
 #48: G T T G A T C C A A G A A A G G A C C C (171-190).

40 When the above primers are in use in PCR, combined use (e.g., of #23 and #25, #32 and #36, or #33 and #48) can enhance the effect of PCR.

This invention also covers NANB hepatitis virus detection systems (e.g., PCR) using the above oligonucleotide primers.

45 Examples of application of this invention are shown below. However, this invention shall in no way be limited to those examples.

Examples

Example 1 - Determination of the nucleotide sequence of the 5' terminus of NANB hepatitis virus:

50 (1) Isolation of RNA

55 RNA was isolated by the method described below from a plasma sample (HC-J1) of a Japanese blood donor who tested positive for HCV antibody and a sample (HC-J4) from a chimpanzee challenged with NANB hepatitis for infectivity but which tested negative for HCV antibody by Ortho HCV Ab ELISA Test (Ortho Diagnostic Systems, Tokyo, Japan).

1.8 ml of each of the plasma samples was added with 1 ml of Tris chloride buffer (10mM, pH 8.0) and centrifuged at 68 x 10³ rpm for 1 hour. The precipitate was suspended in Tris chloride buffer (50 mM, pH 8.0) con-

taining 200 mM NaCl, 10 mM EDTA, 2% (w/v) sodium dodecyl sulfate (SDS) and proteinase K (1 mg/ml), incubated at 60°C for 1 hour, then extracted by phenol/chloroform and precipitated by ethanol to obtain RNAs.

(2) cDNA synthesis

5

RNA isolated from HC-J1 plasma was incubated at 70°C for 1 minute and used as a template. 10 units of reverse transcriptase (cDNA Synthesis Plus, Amersham Japan) and 20 pmol of oligonucleotide primer (20 mer) were added and incubated at 42°C for 1.5 hours to obtain cDNA. Primer #8 (5'- G A T G C T T G C G G A A G C A A T C A - 3') was prepared by referring to the base sequence shown in figure 47-1 (sequence position 401 to 420) of the European Patent Application No. 88310922.5 (the entire application (now European Patent No. 0,318,216) is incorporated by reference).

10

(3) cDNA was amplified by the following Polymerase Chain Reaction (PCR).

15

cDNA was amplified for 35 cycles according to Saiki's method (Science 239, 487-491 (1988)), incorporated by reference in its entirety, using Gene Amp DNA Amplifier Reagent (Perkin-Elmer Cetus) on a DNA Thermal Cycler (Perkin-Elmer Cetus).

(4) Determination of nucleotide sequence by assembling cDNA clones.

20

As shown in Figure 1, the nucleotide sequence of the 5' termini of the genomes of strains HC-J1 and HC-J4 were determined by combined analysis of clones obtained from the cDNA library constructed in bacteriophage lambda gt10 and clones obtained by amplification of HCV specific cDNA by PCR. Figure 1 shows the 5' terminal sequence of NANB hepatitis virus genome together with cleavage sites of restriction endonucleases and sequences of primers used. In Figure 1, solid lines are nucleotide sequences determined by clones from bacteriophage lambda gt10 library while dotted lines show sequences determined by clones obtained by PCR.

25

The 1656 nucleotide sequence of HC-J1, spanning nt437-2092, was determined by the clone ø41 obtained by inserting the cDNA synthesized with the primer #8 into bacteriophage lambda gt10 (Amersham).

30

Primer #25 (5' - T C C C T G T T G C A T A G T T C A C G - 3') of nt 807 - 826 was synthesized based on that ø41 sequence, and 4 clones (ø60, ø61, ø66 and ø75) were obtained to cover the upstream sequence nt1 - 826.

The upstream sequence of strain HC-J1 was determined by clones obtained by PCR using primers #44 (5' - G G C G A C A C T C C A C C A T A G A T - 3') and #25 (5' - T C C C T G T T G C A T A G T T C A C G - 3').

35

The downstream sequence of 1163 nucleotides, from nt721 up to 1883, of strain HC-J4 was determined by 3 clones (C2821, C3173 and C3192) by PCR using primers #30 (5' - C T C A T G G G G T A C A T T C C G C T - 3') and #42 (5' - T C G G T C G T C C C C A C C A C A A C - 3').

From the analysis described above, nucleotide sequences of the 5' termini of the genomes of strains HC-J1 and HC-J4 were determined as shown below.

40

The nucleotide sequence of the genome of strain HC-J1 is shown in line (a) and that of strain HC-J4 in line (b), the latter showing only differing nucleotides vis a vis (a). Noncoding region nt1 - 324 is shown in small letters. nt325 - 1863 is a region coding for various protein starting with initiation codon ATG and is shown in capital letters (the nucleotide sequence in the aforementioned European Patent Application started only with the 1673th nucleotide, and missed the upstream sequence which is originally revealed in this invention):

45

50

55

EP 0 461 863 A1

	(a)	g g c g a c a c t c	c a c c a t a g a t
	(b)	- - - - -	- - - - -
5		c a c t c c c c t g	t g a g g a a c t a
		- - - - -	- - - - -
		c t g t c t t c a c	g c a g a a a g c g
10		- - - - -	- - - - -
		t c t a g c c a t g	g c g t t a g t a t
		- - - - -	- - - - -
15		g a g t g t c g t g	c a g c c t c c a g 100
		- - - - -	- - - - -
		g a c c c c c c c t	c c c g g g a g a g
20		- - - - -	- - - - -
		c c a t a g t g g t	c t g c g g a a c c
		- - - - -	- - - - -
25		g g t g a g t a c a	c c g g a a t t g c
		- - - - -	- - - - -
		c a g g a c g a c c	g g g t c c t t t c
30		- - - - -	- - - - -
		t t g g a t a a a c	c c g c t c a a t g 200
		- - - - -	- - - - -
35		c c t g g a g a t t	t g g g c g c g c c
		- - - - -	- - - - -

40

45

50

55

c c c g c a a g a c l g c l a g c c g a
 - - - - - g - - - - -
 5 g l a g l g l l g g g l c g c g a a a g
 - - - - -
 g c c l l g l g g l a c l g c c l g a l
 10 - - - - -
 a g g g l g c l l g c g a g l g c c c c 300
 - - - - -
 15 g g g a g g l c l c g l a g a c c g l g
 - - - - -
 c a c c A T G A G C A C G A T T C C C A
 20 - - - - - A - - - T -
 A A C C T C A A A G A A A A C C A A A
 - - - - -
 25 C G T A A C A C C A A C C G T C G C C C
 - - - - - C - - - - -
 A C A G G A C G T C A A G T T C C C G G 400
 30 - - - - -
 G T G G C G G T C A G A T C G T T G G T
 - C - - T - - - - -
 35 G G A G T T T A C T T G T T G C C G C C
 - - - - - C - - - - -
 C A G G G G C C C T A G A T T G G G T G
 40 - - - - - C - - G - - - - -
 T C C G C G C G A C G A G G A A G A C T
 - - - - - T - - - - -
 45 T C C G A G C G G T C G C A A C C T C G 500
 - - - - -
 A G C T A G A C G T C A G C C T A T C C
 50 T - - A T - G - - A - - A - - - - -
 - - - - -

EP 0 461 863 A1

	CCAAGGTGCC	TCGGCCCCGAG
	-----CT--	C--A-----
5	GGCAGGACCT	GGGCTCAGCC
	-----G----	-----
	CGGCTACCCCT	TCGGCCCCCTCT
10	-----	-----
	ATGGCAATCA	GGGCTCCGGG600
	-----	-----TG----
15	TGGCGCGGAT	GGCTCCTGTC
	-----A-----	-----
	TCCCCCGTGGC	TCTCGGCCTA
20	A-----C----	--C-----
	GTTGGGGCCC	CACGGACCCC
	-----	-----
25	CGGCGTAGGT	CGCGCAATTT
	-----	-----T--C--
	GGGTAAGGTC	ATCGATACCC700
30	-----	-----
	TCACGTGCGG	CTTCGCGGAC
	-T--A-----	-----T
35	CTCATGGGGT	ACATACCGCT
	-----	-T--T-----
	CGTCGGGGCC	CCTCTTGGAG
40	-----	--C--A--G-
	GGGCTGCCAG	GGCCCTGGCG
	-----	-----T-----A
45	CATGGCGTCC	GGGTTCTGGA800
	--C--T-----	-----
	ACACGGGCTG	AACTATGCAA
50	G-----	-----

55

C A G G G A A C C T T C C T G G T T G C
 - - - - - T - G - - C - - - - -
 5 T C T T T C T C T A T C T T C C T T C T
 - - - - - - - - - - - - - C T -
 G G C C C T G C T C T C T T G C C T G A
 10 - - - T T - - - - G - - C - - T T - - -
 C T G T G C C C G C T T C A G C C T A C 900
 - C A - C - - A - - - - - C - - T - - T
 15 C A A G T G C G C A A C T C C A C A G G
 G - - - - - - - - - - - G T G T - C - -
 G C T T T A T C A T G T C A C C A A T G
 20 - A - A - - C - - - - - - - G - - C -
 A T T G C C C T A A C T C G A G T A T T
 - C - - - T - C - - - - - A - - C - - -
 25 G T C T A C G A G G C G C A C G A T G C
 - - - - - T - - - - - - A G C G - - C A T
 C A T C C T G C A T A C T C C G G G C T 1000
 30 G - - - A - - - - - - - - - C - - - -
 G T G T C C C T T C C G T T C G C G A G
 - C - - G - - C - - - - - - - G - - -
 35 G G C A A C G T C T C G A G G T G T T G
 - A - - - - A G - - - C C - T - - C - -
 G G T G G C G A T G A C C C C C A C G G
 40 - - - A - - - C - C - - T - - - - - C
 T A G C C A C C A G G G A C G G C A A A
 - C - - G G - - - - - A - T - C - - G C
 45 C T C C C C G C G A C G C A G C T T C G 1100
 G - - - - - A - T - - - A C A A - A - -
 A C G T C A C A T C G A T C T G C T T G
 50 - - - C - - - G - - - - C T - - - - C -

| | | | |
|----|-----------------------|--------------------------|--|
| | T C G G G A G C G C | C A C C C T C T G T | |
| | - T - - - G C C - - | T G - T T - - - - C | |
| 5 | T C G G C C C T C T | A C G T G G G G G A | |
| | - - C - - T A - G - | - - - - - - - - - | |
| | T C T G T G C G G C | T C C G T C T T C C | |
| 10 | - - - C - - - - - A | - - T - - T - - - - | |
| | T T A T T G C T C A | A C T G T T T A C C 1200 | |
| | - C G - C T C C - - | G - - - - - C - - - | |
| 15 | T T C T C T C C C A | G G C G C C A C T G | |
| | - - - - - G - - T C | - C - - G - - T G A | |
| | G A C A A C G C A A | G G C T G C A A T T | |
| 20 | - - - - - G T - - - C | - A - - - - - C - | |
| | G T T C T A T C T A | C C C C G G C C A T | |
| | - C - - A - - - - - | T - - - - - - - - - | |
| 25 | A T A A C G G G T C | A T C G C A T G G C | |
| | T - - T - A - - - - | - C - - - - - - - - | |
| | A T C G G A T A T C | A T G A T G A A C T 1300 | |
| 30 | T - - - - - - - - - | - - - - - - - - - | |
| | G G T C C C C T A C | G G C G C C G T T G | |
| | - - - - - A - - - - - | A A - A - - C C - A | |
| 35 | G T A A T G G C T C | A G C T G C T C C G | |
| | - - C G - - T - G - | - - T - - - - - - - | |
| | G A T C C C A C A A | G C C A T C T T G G | |
| 40 | - - - - - - - - - - | - - T G - - G - - - | |
| | A T A T G A T C G C | T G G T G C T C A C | |
| | - C - - - G - G - - | G - - G - - C - - - | |
| 45 | T G G G G A G T C C | T G G C G G G C A T 1400 | |
| | - - - - - - - - - - | - - - - - - - - C - | |
| | A G C C T A T T T C | T C C A T G G T G G | |
| 50 | T - - C - - C - A T | - - - - - - - - A - | |

5 G G A A C T G G G C G A A G G T C C T G
 - - - - - T - - - - -
 G T A G T G C T G T T G C T G T T T G C
 A - T - - - G C - C - A - - C - - C - -
 10 C G G C G T C G A C G C G G A A A C C A
 - - - - - T - - - - G - - - G - - - T
 T C G T C T C C G G G G G A C A A G C C 1500
 A - A C G - - G - - - - - G G C G - - -
 15 G C C C G C G C C A T G T C T G G A C T
 A G - - A - A - - - C C - - C A C G - -
 20 T G T T A G T C T C T T C A C A C C A G
 C - C G T C C - - - - - T - - - - T -
 G C G C T A A G C A G A A C A T C C A G
 25 - G - - G T C T - - - - G A - - - - -
 C T G A T C A A C A C C A A C G G C A G
 - - T G - G - - T - - - - - - - -
 30 T T G G C A C A T C A A T A G C A C G G 1600
 C - - - - - - - - - C - - G - - T -
 C C T T C A A C T G C A A T G A A A G C
 35 - - C - A - - - - - - - - - - C T C -
 C T T A A C A C C G G C T G G T T A G C
 - - C C - - - - T - - G - T C C - T - -
 40 A G G G C T T A T C T A T C A A C A C A
 C - C - - - G T - - - - C A C - - - -
 A A T T C A A C T C T T C G G G C T G T
 45 G G - - - - - - - G - - C - - G - - C
 C C C G A G A C C T T G G C C A G C T G 1700
 - - G - - - C - C A - - - - - - -
 50 C C G A C G C C T T A C C G A T T T T C
 - - - C - C - A - - G A - T G G - - C -


```

      A C C A G G G C T G   G G G C C C T A T C
      C - - - - - A - -   - - - - - C - - -
5     A G T C A T G C C A   A C G G A A G C G G
      - C C T - - A - T G   - G C C T G A - A -
      C C C C G A C C A A   C G C C C C T A T T
10    - - - G - - T - - G   A - G - - T - - - -
      G T T G G C A C T A   C C C C C C A A A A 1800
      - C - - - - - T - -   - G - G - - T C G -
15    C C T T G C G C T A   T C G T G C C C G C
      - - G - - T - - - -   - - - - A - - - -
      A A A G A G C G T A   T G T G C C C C G G
20    G T C - C A G - - G   - - - - - T - - A -
      T A T A T T G C T T   C A C T C C C A G C
      - G - - - - - - - -   - - - C - - A - -
25    C C C 1863
      - - T

```

For the gene region (nt325 - 1863) of strains HC-J1 and HC-J4, the sequences of 513 amino acids encoded were determined and homology, amino acids components and hydrophilicity of the amino acids between the two strains were studied. As a result, region nt325-864 was considered to be coding for the NANB hepatitis virus core proteins. Mutations or differences in the nucleotide sequence in this region were relatively smaller than in other coding regions and approximately 80% of the nucleotide mutations or differences identified were not accompanied by a change in amino acid sequence. Together with the nucleotide sequence of the 5' non-coding region already described above, the sequences of the structural gene region were also helpful in choosing appropriate nucleotide sequences of oligonucleotide primers used for the detection system in this invention. The envelope proteins is considered to be encoded by nucleotides 865-1476 and nonstructural proteins encoded by nucleotides 1477 and above.

The inventors have also identified that among oligonucleotide primers for the core region, the primer #25 (nt807 - 826), which has the least mutations or differences, is the best for the detection system of this invention.

Example 2 - Synthesis of primers and the establishment of the detection system based on the 5' noncoding region and the core protein coding region.

(1) Synthesis of oligonucleotide primers.

Oligonucleotide primers (20 mer) were synthesized based on the 5' noncoding region sequences and the core protein coding region of strains HC-J1 and HC-J4 determined in Example 1. Oligonucleotide primer of HCV was also synthesized according to the nucleotide sequence disclosed in the European Patent Application No. 88310922.5 previously described. The model 3808 DNA Synthesizer (Applied Biosystems Japan) was used for such synthesis.

The number of primers synthesized were 20 (#3, 4, 5, 6, 9, 10, 11, 12, 16, 17, 21, 22, 23, 25, 32, 33, 34, 35, 36 and 48), and the position from the 5' terminus and nucleotide sequence for each of them is shown in Table 1.

(2) Isolation of NANB hepatitis viral RNA from a sample.

1 ml of a plasma sample was centrifuged on a model TL-100 (Beckman) ultracentrifuge at 9×10^4 rpm for

15 minutes and the precipitate thus obtained was suspended in buffer (containing 200 mM NaCl, 10 mM EDTA, 2% (w/v) sodium dodecyl sulfate (SDS) and proteinase K (1 mg/ml)) for incubation at 60°C for 1 hour.

Nucleic acids were extracted twice by using the same volume of phenol/chloroform and precipitated in ethanol at -20°C for over 3 hours. The precipitate was suspended in 70% ethanol for centrifugation and the precipitate was dissolved in 5 µl of distilled water after lyophilization.

(3) cDNA synthesis.

RNA extracted from a plasma sample in (2) above was denatured by heating at 70°C for 1 minute and cooled on ice before synthesis of cDNA. cDNA was synthesized by reverse transcription. 100 pmol each of antisense primers #5, 6, 11, 12, 16, 17, 25, 35, 36 and 48 were added with 4 kinds of deoxyribonucleoside 5'-triphosphates (Takara, Japan), 10 units of RNase Inhibitor (Takara, Japan) and 10 units of Reverse transcriptase AMV (Boehringer Mannheim, Germany), and incubated at 42°C for 90 minutes in a buffer (containing 10 µl of Tris chloride (50 mM, pH 8.4), 8 mM MgCl₂, 30 mM KCl and 1 mM dithiothreitol) to synthesize cDNA. cDNA thus obtained was purified by phenol/chloroform extraction.

(4) Amplification by PCR.

PCR was carried out using DNA Thermal Cycler (Perkin-Elmer Cetus) and DNA Amplification Reagent Kit (Perkin-Elmer Cetus) by the well-known method of Saiki et al. (1988). The reaction cycle of denaturalization (one minute at 94°C), annealing of primers (1.5 minutes at 55°C), and amplification of primers (3 minutes at 72°C) was repeated 35 times.

The PCR product was electrophoresed in a mixed agarose gel of 1-1.5% Nusieve and 1-1.5% Seakem (FMC), and, after staining with ethidium bromide, its bands were confirmed by ultraviolet radiation.

(5) Amplification by second-stage PCR.

The product obtained by the first pair of primers (#32 and #36, for example) by PCR can be subjected to second-stage PCR if necessary. As primers for such PCR, a pair of primers of nucleotides for regions within those of the first pair of primers (#33 and #48, for example) were chosen and PCR reaction cycle was repeated 30 times for 5 µl of the product obtained in the first-stage PCR. Reaction conditions for each cycle was denaturalization (1 minute at 94°C), annealing (1.5 minutes at 55°C), and amplification (2 minutes at 72°C). The product obtained in the second-stage PCR was electrophoresed and analyzed in the method described in (4) above.

Example 3 - Selection of pairs of primers effective for detection of NANB hepatitis virus by PCR.

Results of PCR test with two pairs of primers for 10 samples determined positive for HCV antibody (plasma samples nos. 1, 3, 5, 7 and 9 from Japanese blood donors and serum samples nos. 2, 4, 6, 8 and 10 from NANB hepatitis patients) are shown in Table 2.

PCR amplification was tried for 10 target nucleotide sequence regions; 2 regions each from NS5, NS3, and E-NS1 and its upstream (NS=nonstructural region, E-NS1=nucleotide region bridging the envelope region and nonstructural region number 1) referred to in Chiron's European Application), 2 regions each from the core region and 5' noncoding region identified under this invention.

As a result, when two pairs of primers from the 5' noncoding region (#32/#36 and #33/#48) and one pair of primers from the core region (#23/#25) were used, expected sizes of NANB cDNA bands (242 bp, 145 bp and 377 bp) for respective regions were detected.

In the other 7 regions, however, only 2 to 9 out of 10 samples could successfully be amplified, although the presence of RNA itself was confirmed in each sample. It was therefore concluded that pairs of primers #32/#36 and #33/#48 from the 5' noncoding region, and #23/#25 from the core region, were widely effective for detection of HCV RNA. Thus, selection of primers from NS5, NS3 and E-NS1 regions coding for the nonstructural protein of the virus are quite insignificant.

In some cases, single-stage PCR is sufficient for detection of NANB hepatitis viral RNA. However, to enhance sensitivity, two-stage PCR is recommended.

For example, samples which did not show the expected band of 242 bp when their cDNA was synthesized using #36 primer and amplified by PCR with primers #36 and #32 (first-stage PCR) were then subjected to the second-stage PCR using primers #33 and #48 and the first-stage PCR product as a template (second-stage PCR). If the 145 bp band does not occur after second-stage PCR then the sample did not contain viral RNA.

Example 4

32 samples from chronic NANB hepatitis patients, 10 samples from chronic hepatitis B patients, and 12 samples from blood donors with normal ALT levels were tested for NANB hepatitis virus RNA by PCR. Results are shown in Table 3.

Preliminary test of 32 samples from NANB hepatitis patients showed 20 samples positive and 12 samples negative for anti-HCV. All 10 samples from hepatitis B patients and 12 samples from blood donors with normal ALT levels were negative for anti-HCV.

For the 20 samples out of the 32 total samples from NANB hepatitis patients which tested positive for anti-HCV, RNA was detected in 15 samples by the first-stage PCR and the remaining 5 by the second-stage PCR. Thus 100% of the samples (which tested positive for anti-HCV) tested positive by PCR.

Out of the 12 samples from NANB hepatitis patients which tested negative for anti-HCV, 7 samples by the first-stage PCR and 4 samples by the second-stage PCR (or 92%) turned out to be positive for NANB hepatitis viral RNA. All 10 hepatitis B cases and all 12 blood donor cases with normal ALT levels subjected to the test (so far as the second-stage PCR) were negative for the viral RNA. From these data, NANB hepatitis RNA detection system using oligonucleotide as primers has proven its excellent performance, and its two-stage PCR system in particular has proven its superb performance both in sensitivity (more than 50% higher than anti-HCV and detecting as much as 96.9% of NANB hepatitis viral RNA) and specificity.

(5) Sensitivity of the detection system for NANB hepatitis virus by cDNA/two-stage PCR.

Sensitivity of NANB hepatitis virus detection system by cDNA/two-stage PCR under this invention is described below. Results are shown in Table 4.

10-fold serially diluted samples of plasma (having known infectious unit of 10^7 CID/ml) were prepared and tested 3 times. In the first-stage PCR, the expected band of 242 bp was confirmed for 100 CID/ml in two tests and as low as 10 CID/ml in the remaining one test.

In the second-stage PCR, the expected band of 145 bp was confirmed for 10 CID/ml in two tests and as low as 1 CID/ml in one test. No band was detected for concentrations less than 1 CID/ml or for negative samples.

Average titer of NANB hepatitis patients is estimated to be 10^{2-4} and the described detection system is considered to give clinically significant sensitivity for diagnosis of NANB hepatitis patients.

The present invention thus provides a highly sensitive and specific detection system for NANB hepatitis virus. Accordingly, this invention will become instrumental in accurate diagnosis of hepatitis patients and screening of donor blood for prevention of posttransfusion hepatitis.

The present invention also concerns a nucleotide sequence of NANB which contains at least a portion of the bases 1-1863 described above. The sequence is constituted of a plurality of nucleotides and contains at least one primer as shown in table 1. In addition, the sequence is terminated at least at one end with a primer as shown in table 1.

The present invention further concerns a method of detecting non-A, non-B hepatitis virus comprising:

- (1) synthesizing cDNA from viral RNA;
- (2) amplifying said cDNA by PCR in first stage to produce a product;
- (3) amplifying the product by PCR in a second stage.

The amplifying is carried out by using at least one oligonucleotide primer according to claim 8. The primer in step (1) may be a pair of primers and the primer in step (2) may be a pair of primers from a region within the pair of primers in step (1). The pair of primers in step (1) may be primers #32 and #48 in table 1 and the pair of primers in step (2) may be primers #33 and #48 in table 1. The pairs of primers #32/#36, #33/48, and #23/#25 in table 1.

The present invention also concerns diagnostic test kits for detection NANB in biological samples, including for example blood and serum samples. The test kit includes (1) at least one primer derived from the nucleotide sequence disclosed above, (2) dATP, dTTP, dGTP, and dCTP; and (3) heat stable DNA polymerase. Kits suitable for diagnosis and NANB and containing the appropriate reagents are constructed by packaging the appropriate materials, including the primer in suitable containers, along with the remaining reagents and materials required, as well as a suitable set of instructions for conducting the test.

Further variations and modifications of the invention will become apparent to those skilled in the art from the foregoing and are intended to be encompassed by the claims appended hereto.

Japanese Patent Application No. Heisei 2 Nen 153402, filed on June 12, 1990, is relied on and incorporated by reference.

5

10

15

20

25

30

35

40

45

50

| Primers. | Nucleotide Position | Nucleotide Sequences |
|----------|---------------------|-----------------------|
| # 3 | 126-145 | AAACCTTGCGGTATTGTGCC |
| # 4 | 153-172 | AGTGTGTGTGGTCCGGTATA |
| # 5 | 268-287 | CGGTGGCCTGGTATTGTTAA |
| # 6 | 303-322 | GAGTTCATCCAGGTACAACC |
| # 9 | 6427-6446 | AGATGGCTTTGTACGACGTG |
| #10 | 6490-6509 | TCCAATACTCACCAGGACAG |
| #11 | 6761-6780 | CACAGCTAGTTGTCAGTACG |
| #12 | 6786-6805 | TTGATGTAGCAAGTGAGGGT |
| #16 | 4029-4048 | CTGGTGACAGCAGCTGTAAA |
| #17 | 4061-4080 | TGAAGAGGAGGGTTTGGCTA |
| #21 | 3669-3688 | TATTGCCTGTCAACAGGCTG |
| #22 | 3759-3778 | CGAGAGTTCCGATGAGATGGA |
| #23 | 450-469 | TAGATTGGGTGTGCGCGCGA |
| #25 | 807-826 | TCCCTGTTGCATAGTTCACG |
| #32 | 7-26 | ACTCCACCATAGATCACTCC |
| #33 | 46-65 | TTCACGCAGAAAGCGTCTAG |
| #34 | 475-494 | AAGACTTCCGAGCGGTCGCA |
| #35 | 568-587 | TTGCCATAGAGGGGCCAAGG |
| #36 | 229-248 | AACACTACTCGGCTAGCAGT |
| #48 | 171-190 | GTTGATCCAAGAAAGGACCC |

55

Table 2: Detection of NANB hepatitis viral RNA in HCV antibody positive samples by PCR using various sets of primers.

| Primers
Sample No. | 5' noncoding Region | | presumable core gene | | E-NS 1 | | NS 3 | | NS 5 | |
|-----------------------|----------------------------|----------------------------|----------------------------|----------------------------|--------------------------|--------------------------|----------------------------|----------------------------|---------------------------|----------------------------|
| | #32
/
#36
(242bp) | #33
/
#48
(145bp) | #23
/
#25
(377bp) | #34
/
#35
(113bp) | #3
/
#6
(197bp) | #4
/
#5
(135bp) | #21
/
#17
(412bp) | #22
/
#16
(290bp) | #9
/
#12
(379bp) | #10
/
#11
(291bp) |
| 1 | + | + | + | + | + | + | + | + | + | + |
| 2 | + | + | + | + | - | + | + | + | + | - |
| 3 | + | + | + | + | - | + | + | + | + | - |
| 4 | + | + | + | + | - | + | - | + | + | - |
| 5 | + | + | + | + | - | - | - | + | + | - |
| 6 | + | + | + | + | - | + | + | + | + | - |
| 7 | + | + | + | + | - | - | - | + | + | - |
| 8 | + | + | + | + | - | - | - | + | + | - |
| 9 | + | + | + | - | - | + | - | - | - | - |
| 10 | + | + | + | - | - | + | - | - | - | - |

Samples Nos. 1, 3, 5, 7 and 9 are taken from blood donors, and samples Nos. 2, 4, 6, 8 and 10 are taken from chronic NANB hepatitis patients.

Table 3: NANB hepatitis viral RNA detection by cDNA/two-stage PCR.
One pair each of primers #32 and 36, and #33 and #48 was used for the first-stage and second-stage PCR respectively.

| Source of Samples | Total number of Samples | Number of samples positive for anti-HCV by ORTHO's EIA | Number of samples positive for NANB hepatitis viral RNA | | |
|------------------------------------|-------------------------|--|---|---------------|------------|
| | | | 1st-stage PCR | 2nd-stage PCR | Total (%) |
| Chronic NANB hepatitis | 32 | 20 (62.5%) | 22 | 9 | 31 (96.9%) |
| Chronic hepatitis B | 10 | 0 (0) | 0 | 0 | 0 (0) |
| Blood donors with normal ALT level | 12 | 0 (0) | 0 | 0 | 0 (0) |

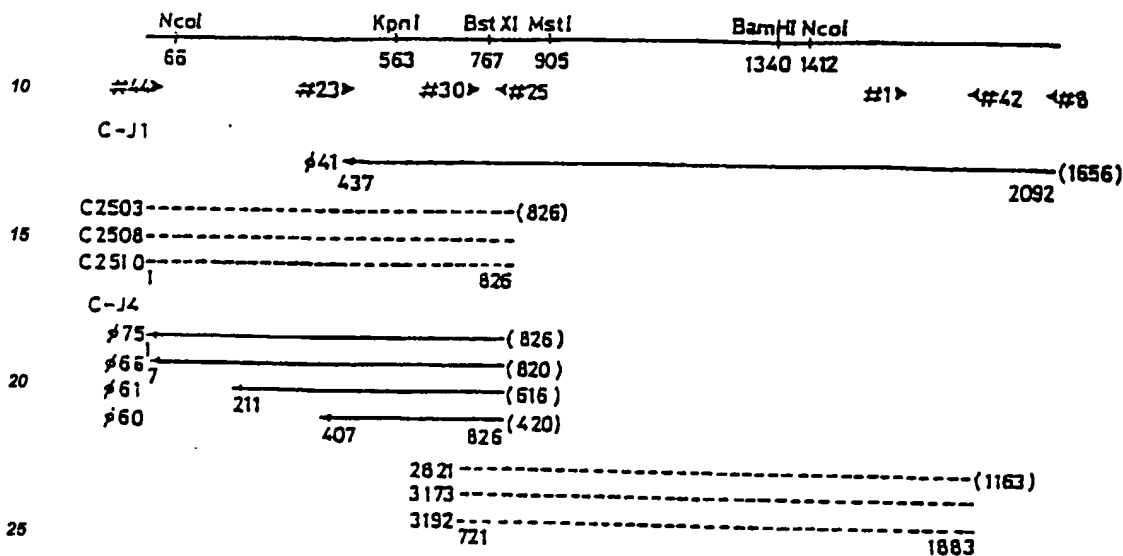
Table 4: Detection of NANB hepatitis viral RNA by two-stage PCR in samples with known infectivity titers. One pair each of primers #32 and #36, and #33 and #48 were used for the first-stage and second-stage respectively.

| Test | P C R | *Serial Dilution (CID/ml) | | | | | | | | Control
(Negative) |
|----------------|-----------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------|--------------------|----------------------|---|-----------------------|
| | | $\times 10^2$
(10^5) | $\times 10^3$
(10^4) | $\times 10^4$
(10^3) | $\times 10^5$
(10^2) | $\times 10^6$
(10) | $\times 10^7$
1 | $\times 10^8$
0.1 | | |
| First
Test | 1st-stage | +++ | +++ | ++ | + | - | - | - | - | |
| | 2nd stage | NT | NT | NT | + | + | - | - | - | |
| Second
Test | 1st-stage | +++ | +++ | ++ | + | +/- | - | - | - | |
| | 2nd-stage | NT | NT | NT | + | + | + | - | - | |
| Third
Test | 1st-stage | +++ | +++ | ++ | + | - | - | - | - | |
| | 2nd-stage | NT | NT | NT | + | + | - | - | - | |

*Some plasma negative for HCV antibody and HCV RNA was used as diluent and control. CID means Chimpanzee Infectious Dose.

Fig. 1

5



30

Claims

1. A nucleotide sequence of non-A, non-B hepatitis virus comprising [bases 1-1863].
2. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 1-1672].
3. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 1-324].
4. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 3, wherein the 187th nucleotide A is replaced by C, the 217th nucleotide C is replaced by T, and the 226th nucleotide A is replaced by G.
5. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 325-1863].
6. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 325-864].
7. The nucleotide sequence of non-A, non-B hepatitis virus according to claim 1, comprising [bases 865-1476].
8. An oligonucleotide primer derived from the nucleotide sequence of non-A, non-B hepatitis virus according to claim 1.
9. The oligonucleotide primer according to claim 8, wherein said primer contains from 15 to 25 nucleotides.
10. The oligonucleotide primer according to claim 9, wherein said primer contains 20 nucleotides.
11. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #23 in table 1.

12. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #25 in table 1.
13. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #32 in table 1.
- 5 14. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #33 in table 1.
15. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #36 in table 1.
16. The oligonucleotide primer according to claim 8, wherein said primer comprises primer #48 in table 1.
- 10 17. An oligonucleotide primer derived from the nucleotide sequence of non-A, non-B hepatitis virus according to claim 3.
18. An oligonucleotide primer derived from the nucleotide sequence of non-A, non-B hepatitis virus according to claim 7.
- 15 19. A method of detecting non-A, non-B hepatitis virus comprising synthesizing of cDNA from viral RNA and amplifying said cDNA by PCR using at least one oligonucleotide primer according to claim 8.
- 20 20. The method according to claim 19, wherein said oligonucleotide primer is selected from the group consisting of: primer #23 in table 1, Primer #25 in table 1, primer #32 in table 1, primer #33 in table 1; primer #36 in table 1, and primer #48 in table 1.
- 25 21. The method according to claim 20, wherein said oligonucleotide primers consists of primer #32 in table 1 and primer #36 in table 1.
22. The method according to claim 20, wherein said oligonucleotide primers consists of primer #33 in table 1 and primer #48 in table 1.
- 30 23. The method according to claim 20, wherein said oligonucleotide primers consists of primer #23 in table 1 and primer #25 in table 1.
24. An oligonucleotide primer derived from the nucleotide sequence of non-A, non-B hepatitis virus according to claim 6.
- 35 25. A test kit for diagnosing non-A, non-B hepatitis or for detecting non-A, non-B hepatitis virus, said kit comprising:
 - 1) at least one primer according to claim 8;
 - 2) dATP, dTTP, dGTP, and dCTP; and
 - 40 3) heat stable DNA polymerase.
26. A nucleotide sequence of non-A, non-B hepatitis virus comprising at least a portion of the bases 1-1863, said sequence being constituted of a plurality of nucleotides and containing at least one primer as shown in table 1.
- 45 27. A nucleotide sequence of non-A, non-B hepatitis virus comprising at least a portion of the bases 1-1863, said sequence being constituted of a plurality of nucleotides and terminated at least at one end with a primer as shown in table 1.
- 50 28. A method of detecting non-A, non-B hepatitis virus comprising:
 - (a) synthesizing cDNA from viral RNA;
 - (2) amplifying said cDNA by PCR in a first stage to produce a product;
 - (3) amplifying said product by PCR in a second stage; said amplifying being carried out by using at least one oligonucleotide primer according to claim 8.
- 55 29. The method according to claim 28, wherein said primer in step (1) is a pair of primers and said primer in step (2) is a pair of primers, wherein said pair of primers in step (2) are from a region within the pair of primers in step (1).

30. The method according to claim 29, wherein said pair of primers in step (1) are primers #32 and #48 in table 1 and said pair of primers in step (2) are primers #33 and #48 in table 1.

5 31. The method according to claim 28, wherein said pairs of primers are selected from pairs of primers #32/#36, #33/#48, and #23/#25 in table 1.

10

15

20

25

30

35

40

45

50

55

European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | EP 91305270.0 |
|--|---|---|--|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.5) |
| P, X | EP - A1 - 0 388 232
(CHIRON CORPORATION)
* Abstract; claims 15-18 * | 1, 3, 6,
7, 25,
26, 28 | C 07 H 21/04
C 12 Q 1/70
C 12 Q 1/68
C 12 N 15/51
C 12 P 19/34 |
| D, A | EP - A1 - 0 318 216
(CHIRON CORPORATION)
* Claims 1-5, 21, 29 * | 26, 28 | |
| P, A | EP - A2 - 0 398 748
(CHIRON CORPORATION)
* Abstract; claims 1-4, 7, 15, 16, 18 * | 1, 3, 8,
19, 25,
28 | |
| A | WO - A1 - 90/00 597
(GENELABS INCORPORATED)
* Abstract; claims 10-13, 27 * | 1, 19,
28 | |
| A | CHEMICAL ABSTRACTS, vol. 110,
no. 25, June 19, 1989,
Columbus, Ohio, USA
QU. L. CHOO et al. "Isolation
of cDNA clone derived from a
blood-borne non-A, non-B viral
hepatitis genome"
page 145; column 1, abstract-
no. 226 247n
& Science (Washington D.C.,
1881-) 1989, 244(4902), 359-62
----- | 1 | TECHNICAL FIELDS
SEARCHED (Int. Cl.5)

C 07 H
C 12 Q
C 12 N
C 12 P |
| The present search report has been drawn up for all claims | | | |
| Place of search
VIENNA | Date of completion of the search
12-09-1991 | Examiner
SCHNASS | |
| CATEGORY OF CITED DOCUMENTS
X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document | | T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document | |

EPO FORM (ISO 6593:1990)